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Epigallocatechin-3-gallate modulates antioxidant defense enzyme expression in murine submandibular and pancreatic exocrine gland cells and human HSG cells

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Abstract

Sjogren's syndrome (SS) and type 1 diabetes are prevalent autoimmune diseases in the United States. We reported previously that Epigallocatechin-3-gallate (EGCG) prevented and delayed the onset of autoimmune disease in NOD mice, a model for both Sjogren's syndrome (SS) and type 1 diabetes. EGCG also normalized the levels of proteins related to DNA repair and antioxidant activity in NOD.B10.Sn-H2 mice, a model for primary SS, prior to disease onset. The current study examined the effect of EGCG on the expression of antioxidant enzymes in the submandibular salivary gland and the pancreas of NOD mice and cultured human salivary gland acinar cells. NOD mice consuming 0.2% EGCG daily dissolved in water showed higher protein levels of peroxiredoxin 6 (PRDX6), a major antioxidant defense protein, and catalase, while the untreated NOD mice exhibited significantly lowered levels of PRDX6. Similarly, pancreas samples from water-fed NOD mice were depleted in PRDX6 and superoxide dismutase, while EGCG-fed mice showed high levels of these antioxidant enzymes. In cultured HSG cells EGCG increased PRDX6 levels significantly, and this was inhibited by p38 and JNK inhibitors, suggesting the EGCG-mediated increase in protective antioxidant capacity is regulated in part through MAPK pathway signaling. This mechanism may explain the higher levels of PRDX6 found in EGCG-fed NOD mice. These preclinical observations warrant future preclinical and clinical studies to determine whether EGCG or green tea polyphenols could be used in novel preventive and therapeutic approaches against autoimmune diseases and salivary dysfunction involving oxidative stress.

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Keywords

EGCG; peroxiredoxin 6; NOD; antioxidant defense enzymes; Sjogren's syndrome; Salivary gland

INTRODUCTION

In the United States, 8% of the population is affected by autoimmune diseases, including Sjogren's syndrome (SS) and type 1 diabetes, and 78% of those affected are females (1). Autoimmune-associated lymphocytic infiltration into the salivary and lacrimal glands in the case of SS, and in the pancreatic islets of β cells in type-1 diabetes, is associated with salivary and lacrimal dysfunction (primary SS), or loss of insulin production (type-1 diabetes).

Although the pathogenesis of autoimmune disorders are still not understood fully, oxidative stress has been identified as one of the major contributors to tissue damage in the secretory glands affected by autoimmune reactions (2–5). A role for oxidative stress in SS has been implicated by chemical signatures for reactive oxygen species (ROS) damage to DNA, lipids and proteins observed in labial salivary gland ductal cells from patients with SS, but not in healthy controls (6). Similarly, oxidative stress and reduced antioxidant defense are closely associated with diabetes mellitus (7, 8). In addition to the inflammatory infiltrate, a second source of oxidative stress in glandular epithelium could be alterations in the levels of pro- and anti-oxidant enzymes. Consistent with this, xanthine oxidase levels are increased, and levels of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) decreased, in the conjunctival epithelium of patients with SS (9). Interestingly, SS-A/Ro 52, a major autoantigen marker for SS and systemic lupus erythematosus (SLE), is translocated from the cytoplasm to the nucleus in response to hydrogen peroxide treatment *via* ERK MAPK pathway signaling, suggesting it might be a component in an oxidative (hydrogen peroxide) stress response (10).

We reported previously that polyphenols extracted from green tea leaves and epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea leaves, prevented and delayed the onset of the autoimmune disorders, and reduced the severity of symptoms, in non-obese diabetic (NOD) mice, a mouse model for SS and type 1 diabetes (11, 12). Recently, we found that in a mouse model for primary Sjogren's syndrome (NOD.B10.Sn-H2), peroxiredoxin 6 is significantly decreased in both the salivary gland and pancreas prior to the onset of autoimmune symptoms, and EGCG normalizes the antioxidant defense protein levels (13). However, the mechanism underlying the protective role EGCG plays in these animal models is not clear.

Green tea polyphenols (GTPs) are potent antioxidants, which could potentially serve as a mode of action against autoimmunity, given the role of ROS in autoimmunity and glandular dysfunction (14). On the other hand, it is likely the ROS scavenging ability of GTPs cannot sufficiently explain a dominant mechanism behind the beneficial effects of GTPs in the NOD mouse model, since clinical trials of other antioxidants (N-acetylcysteine-NAC, or vitamin C) for SS treatment have shown little, if any, benefit (15, 16). The effects of GTPs

(direct or indirect) on ROS production in salivary glands and the pancreas have not been assessed, although it is anticipated that it would be significantly reduced.

Despite the non-toxic nature of these phytochemicals, clinical trials of GTPs for the treatment of SS or salivary dysfunction have not been reported. We showed previously that GTPs mediate some of their beneficial effects *via* the p38 and JNK mitogen-activated protein kinase (MAPK) signaling pathways (17). The MAPK signaling pathways are also involved in immune cell function such as tumor necrosis factor (TNF)- α -induced cytotoxicity. Based on this association, MAPK inhibitors (specifically pharmacologic p38 inhibitors), have been developed as potential immunomodulators for treatment of autoimmune-induced inflammation. Unfortunately, the outcomes of clinical trials have largely been disappointing, possibly because these potent inhibitors not only block the abnormal functions of the immune cells, but also the normal functions of the targeted tissues, and the latter require p38 MAPK signaling for normal activities such as secretion of fluid or proteins (reviewed in 18 and 19).

The current study examined salivary gland and pancreatic samples from NOD mice that either consumed water only or consumed 0.2% EGCG-water, to determine the expression levels of antioxidant defense enzymes in tissues affected respectively by Sjogren's Syndrome-like pathology and type I diabetes. In addition, human HSG cell line salivary gland cells were treated with EGCG in combination with MAPK inhibitors to determine the expression levels of the intracellular antioxidant defense enzyme PRDX6.

Materials and Methods

Chemicals and Antibodies

EGCG (>95%) was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-human/rodent superoxide dismutase (SOD) antibody, rabbit anti-human/rodent peroxiredoxin 6, rabbit anti-human/rodent catalase, and goat anti-human actin polyclonal antibody (I-19), were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Pharmacological inhibitors for p38 (SB 203580) and JNK (SP600125) were supplied by EMD Bioscience, Inc., San Diego, CA.

Animal samples—Submandibular salivary gland and pancreas samples were obtained from a study described previously (12). Briefly, salivary gland and pancreas samples were obtained at 22 weeks of age from NOD mice fed with water or 0.2% EGCG-water from the age of 4 weeks. Five micron-thick (5 μ m) serial sections were cut from the paraffin-embedded samples and immunostained. The antioxidant capacity, represented by the proportion of cells expressing peroxiredoxin 6, superoxide dismutase 1 (SOD1), glutathione peroxidase 1, and catalase, was measured by immunohistochemistry.

Immunohistochemical Analysis

Immunohistochemical staining was performed using a standard protocol with Histo-plus Kits (ZYMED Laboratories, CA, U.S.A.) according to the manufacturer's directions. Deparaffinized sections were immersed in methanol containing 3% hydrogen peroxide for 20 min. The sections were incubated with polyclonal antibodies against catalase, SOD,

glutathione peroxidase 1 or PRDX6 overnight at 4°C. The sections were then incubated with the appropriate biotinylated secondary antibody for 10 min and HRP-streptavidin for 10 min. Peroxidase staining was performed for 3~7 min using a solution of DAB chromogen. The sections were counterstained with 0.5% methyl green. For quantification, at least 1000 cells were counted from each slide, and the percentage of cells showing positive nuclear staining was designated as a specific protein index.

Cell culture and treatment

The human salivary gland adenocarcinoma cell line HSG was derived from intercalated ductal epithelium (20). This cell line was maintained in DMEM/Ham's F12 medium, with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml hydrocortisone at 37°C with 5% CO₂.

EGCG was dissolved in cell culture medium as a 50 mM stock immediately before use in the cell culture media. MAPK inhibitors were dissolved in DMSO as a 10 mM stock prior to use in the cell culture media 60 min prior to the addition of EGCG or NAC. Cells were treated with EGCG, MAPK inhibitors, NAC, and combinations of the agents for 24 h prior to cell lysate collection and Western analysis. Cells treated H₂O₂ were exposed to H₂O₂ for 30 min before washed by PBS, and fresh medium was added with appropriate agents (e.g. EGCG or NAC).

Western Blot

After cell treatment, cells were washed in ice-cold PBS and lysed for 20 min in RIPA buffer containing 1 % (v/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 10 µg/ml leupeptin, 3 µg/ml aprotinin and 100 mM phenylmethylsulfonyl fluoride (PMSF). Samples of lysates containing the same amount of protein were loaded in each lane (we used 5–30 µg, depending on the antibody used) and electrophoretically separated on a 12% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon™-P, Millipore Corporation, Bedford, MA). The membrane was blocked for 1 h with 5 % (w/v) non-fat dry milk powder in PBST (0.1% Tween-20 in PBS) and then incubated for 1 h with primary antibody diluted in PBST/milk (rabbit anti-PRDX6 or goat anti-actin). The membrane was washed three times with PBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Experiments were repeated three times with similar results and identical patterns.

Statistical Analysis

All data are reported as mean ± SD or + SEM. Following a D'Agostino and Pearson omnibus normality test, one-way ANOVA with Tukey's or Dunnett's post-test for multiple comparisons or a two-tailed Student's *t* test was used to analyze statistical significance, as appropriate. Differences were considered statistically significant at *p*<0.05.

RESULTS

EGCG elevated antioxidant enzyme levels in NOD mice

By 22 weeks of age, the submandibular glands of NOD mice show changes consistent with a Sjogren's Syndrome-like pathology. Immunohistochemistry was used to determine the proportion of cells expressing selected antioxidant enzymes in the submandibular salivary glands of NOD mice fed either water or EGCG. In the submandibular gland, the proportion of cells expressing PRDX6 in the epithelium was significantly lower in NOD mice (21.7 ± 4.6 (SEM) % positive cells) at 22 weeks of age, in comparison to NOD animals fed with EGCG (41.3 ± 18.1 (SEM)% positive cells, $p=0.0102$) (Figure 1A, 1C, and 1E). Thus, EGCG significantly increased the level of PRDX6 in 22 week NOD mice.

The proportions of submandibular gland cells expressing catalase in NOD mice fed water ($9.8 \pm$ (SEM) 1.5% positive cells) is significantly lower than that in EGCG-fed mouse samples ($14.1 \pm$ (SEM) 1.2% positive cells %, two tailed t test, $p=0.0315$) (Fig 1B, 1D, and 1F).

In contrast to these results, glutathione peroxidase 1 levels in the submandibular gland of NOD mice were unchanged in animals fed EGCG in comparison to animals fed water (7.46 ± 1.63 and $6.13 \pm 1.21\%$ respectively; $p=0.5147$, t -test). Similarly, SOD1 levels in water and EGCG fed mice (2.79 ± 0.71 and $5.53 \pm 1.17\%$ respectively) were not significantly different ($p=0.0570$, t -test, data not shown).

At 22 weeks of age, NOD mice have developed autoimmune type I diabetes, with pathology of the pancreas. At this age, levels of PRDX6 in the pancreatic cells in water-fed NOD mice were very low ($0.3 \pm 0.32\%$). Treatment with EGCG drastically increased PRDX6 levels in NOD mice ($69.4 \pm 5.15\%$, two tailed t test, $p<0.0001$) (Fig 2A, 2C, and 2E). SOD1 showed a similar pattern. SOD1 was almost absent from the pancreatic cells in water-fed NOD mice ($0.1 \pm 0.18\%$). The level of pancreatic SOD1 in NOD mice was significantly elevated by inclusion of EGCG in the drinking water (70.0%; two tailed t test, $p<0.0001$; Fig 2B, 2D and 2F). In the pancreas, there was no significant difference ($p>0.43$; ANOVA) in catalase levels between NOD-water (4.1%) and NOD-EGCG mice (4.1%) (data not shown). Glutathione peroxidase 1 was almost absent in all groups (0.1%) (data not shown). Collectively, these results show that levels of antioxidant protein are reduced overall in the submandibular glands and pancreas of NOD mice at an age where they have developed overt autoimmune disease, and that EGCG can induce higher levels.

EGCG up-regulated PRDX6 protein levels in HSG cells

Figure 3 shows that in HSG cells, 12 h after addition to media, EGCG at 10 and 25 μ M increased PRDX6 protein expression by more than 3 fold (3.51 and 3.21 respectively, $n=3$, $p<0.0026$; ANOVA, Dunnett's post-test) relative to control, while 50 μ M EGCG has no significant impact (1.25 fold, $n=3$, $p=0.89$). At 24 and 36 h, 10 and 25 μ M EGCG induced a more than 2 fold increase of PRDX6 (2.89 and 2.66 fold, 2.38 and 2.48 fold, $n=3$, $p<0.003$), while 50 μ M EGCG still did not show a statistically significant increase (1.06 and 1.59 fold, $p>0.13$, ANOVA, Dunnett's post-test). Assessment of cytotoxicity using the MTT assay demonstrated that EGCG at 10 and 25 μ M for 24 h showed no difference to untreated

control, while EGCG at 50 μ M showed slight cytotoxicity (93.3% of control; ANOVA $p < 0.001$; Dunnett's post-test, data not shown, submitted for publication elsewhere). Therefore, physiological levels of EGCG (25 μ M), but not higher levels, can induce PRDX6 protein levels significantly, and the profile of induction showed no significant differences between the timepoints examined (two-way ANOVA).

PRDX6 expression in response to EGCG and combination of MAPK inhibitors

At 24 h post treatment, the p38 MAPK inhibitor at 10 μ M blocked the inductive effect of 10 and 25 μ M EGCG on PRDX6 (Fig 4, $n=3$, ANOVA $p > 0.7$ Dunnett's post-test in comparison to untreated control). Following treatment with 50 μ M EGCG, the p38 inhibitor had no effect on the level of PRDX6, which was not significantly different from the untreated control ($p > 0.9$). The MTT cytotoxicity assay showed a statistically significant increase in viability relative to control (119%) at 10 μ M EGCG + p38 inhibitor (ANOVA $p = 0.0066$; Dunnett's post-test in comparison to untreated control, data not shown). A similar increase in viability (118%) was seen at 25 μ M EGCG + p38 inhibitor, but this was not significant ($p = 0.1057$, data not shown). Therefore, the lack of significant induction of PRDX6 by EGCG at these doses was not due to cytotoxic reduction of cell numbers. Viability at 50 μ M EGCG + p38 inhibitor (92.9%) was not significantly different from control ($p = 0.3878$, data not shown).

At 24 h post treatment, the JNK MAPK inhibitor at 10 μ M also blocked the inductive effect of 10 and 25 μ M EGCG on PRDX6 (Fig 4, $n=3$, ANOVA $p > 0.2781$ in comparison to untreated control). At 50 μ M EGCG, pre-treatment with the JNK inhibitor significantly lowered the level of PRDX6 below the control value (43.1% $n=3$, $p = 0.0129$). Thus, the induction of PRDX6 by physiological levels of EGCG required active p38 and JNK signaling pathways.

The MTT cytotoxicity assay showed a statistically significant increase in viability (116 and 115%) at 10 and 25 μ M EGCG + JNK inhibitor (ANOVA $p < 0.0001$; Dunnett's multiple comparisons test, data not shown), while 50 μ M EGCG + JNK inhibitor showed a modest, but statistically significant, decline in viability (91.5% compared to control; $p = 0.0029$, data not shown). However, the 56.9% decline in PRDX6 due to treatment with 50 μ M EGCG + JNK inhibitor was significantly more than the 8.5% decline in viability ($p = 0.0006$; two-tailed Student's t-test, data not shown). Therefore, the majority of the decline in PRDX6 below control values was the result of inhibition of JNK pathway signaling, rather than an effect on cell viability as measured by the MTT assay.

Hydrogen peroxide down-regulated PRDX6 expression with or without EGCG or NAC

An initial examination of the role of oxidative stress in the down-regulation of PRDX6 was performed using hydrogen peroxide treatment of HSG cells. HSG cells were incubated with H_2O_2 for 30 min and continued incubation with or without 10 μ M EGCG or NAC for 24 h. Fig 5 demonstrates that at 100 μ M, H_2O_2 significantly lowered PRDX6 expression to 40% in comparison to control ($n=3$, two tailed t test, $p = 0.0036$), regardless of the presence of EGCG (42% vs. control, $n=3$, two tailed t test, $p = 0.0030$), or NAC (35% vs. control, $n=3$, two tailed t test, $p = 0.0071$). That is, this high level of ROS inhibited PRDX6, and neither

EGCG nor NAC at physiological levels reversed the effect to a detectable extent. At 50 μ M, H_2O_2 blocked EGCG-induced PRDX6 expression (98% vs. control, n=3, two tailed t test, p=0.89). In contrast to EGCG, 10 μ M of NAC alone failed to induce PRDX6 expression (95% vs. control, n=3, two tailed t test, p=0.68). These results show that PRDX6 levels can be reduced by elevated levels of H_2O_2 .

DISCUSSION

The NOD mouse model has been a reliable system for the study of human SS and type-1 diabetes (insulin-dependent). We and others reported previously that EGCG delayed/prevented the onset of spontaneous insulin-dependent diabetes in NOD mice (12, 21). Previously, we examined levels of antioxidant proteins in the NOD.B10.Sn.H2 mouse, a model for pSS that does not develop diabetes at early time points, before the onset of frank lymphocytic infiltration (13). In the submandibular gland, the levels of PRDX6 (an antioxidant enzyme) began to decline around 10 weeks, and were significantly lower at 14 weeks, but were restored to normal by dietary EGCG. In contrast, levels of catalase and SOD did not show significant changes. Similarly, in the pancreas, PRDX6 was significantly reduced at 8 weeks, whereas levels were unchanged in EGCG-fed mice. These experiments highlighted PRDX6 as a potential target involved in the beneficial protective effects of EGCG. This indirect provision of antioxidant activity, (rather than a direct antioxidant effect), would be consistent with ROS involvement in autoimmune disorders but no marked benefit from direct antioxidants.

Here we show that in the NOD mouse at 22 weeks of age, after the onset of lymphocytic infiltration and frank autoimmune disease, submandibular gland levels of PRDX6 are significantly lowered, and these levels are increased significantly by dietary EGCG (Fig 1A, 1C, and 1E). In contrast to the NOD.B10.Sn.H2 mouse at 14 weeks, levels of catalase, but not SOD, were also reduced in the NOD mouse at 22 weeks, and were slightly but significantly increased in EGCG fed mice (Fig 1B, 1D and 1F). However, in the pancreas, levels of both PRDX6 and SOD were dramatically lower in the 22 week NOD mice, and these levels were significantly increased by dietary EGCG (Fig 2). Unlike the submandibular gland, levels of catalase were unchanged in the pancreas of NOD mice.

The reduced levels of antioxidant enzymes in the pancreas seen here are consistent with previously reports examining NOD mice (22), and diabetic rats induced by streptozotocin (23).

Collectively, these observations are consistent with the association of ROS and autoimmune disease-affected tissues being caused, in part, by decreased expression of certain key antioxidant enzymes, with the particular enzymes being specific to each tissue. To the best of our knowledge, the restoration of high levels of antioxidant enzymes in the pancreas by EGCG after the onset of disease has not been reported elsewhere. Thus, EGCG could potentially maintain the expression of SOD, catalase, and PRDX6 antioxidant enzymes in humans with autoimmune disorders such as type 1 diabetes and Sjogren's syndrome.

In contrast to catalase and SOD, PRDX6 was decreased in both the submandibular gland and the pancreas. Previously, we showed that levels of PRDX6 were reduced before those of the other two enzymes (13). This suggests that PRDX6 plays a key role in maintaining the antioxidant capacity in exocrine glands, consistent with a known role in providing defensive activity against oxidative stress (24). PRDX6 reduces ROS-mediated cytotoxicity caused by TNF- α and nuclear factor (NF)- κ B activation, and protects retinal ganglion cells from TNF- α - and glutamate-induced apoptosis (27). Cataractous lenses show a 10-fold reduction in PRDX6 expression, and reintroduction of PRDX6 reverses the pathological changes and reduces levels of TGF- β 1, which is associated with certain degenerative diseases (29). PRDX6 transgenic mice are significantly more resistant to ROS-mediated cytotoxicity in comparison to wild type and PRDX6 null mice (28), while PRDX6 knockout mice show impaired wound healing mechanisms after injury or UV-induced damage (25, 26). Collectively, these data suggest that decreased expression levels of PRDX6 could precede autoimmune pathological changes in the glands, and may be involved in the development of overt disease. This warrants further exploration.

Proteomic studies have shown that the expression of PRDX6 is positively regulated by the MAPK pathways, shown previously to be involved in mediating various effects of EGCG (30–33). For example, the JNK and p38 MAPK pathways regulate the caspase 14 gene (17). In the HSG cell line, we found here that EGCG at 10 and 25 μ M significantly induced PRDX6 (2.38–3.51-fold) throughout a 36-hour period (Fig 3). These concentrations are within range of the maximum serum concentration of EGCG, which is below 25 μ M (34). This EGCG-induction of PRDX6 was blocked by inhibitors of JNK or p38 MAPK signaling (Fig 4), consistent with signal transduction of EGCG regulation of PRDX6 via JNK and p38 MAPK pathways. Interestingly, treatment with 50 μ M EGCG did not induce PRDX6, and the JNK inhibitor (but not the p38 inhibitor) lowered PRDX6 levels significantly in the presence of 50 μ M EGCG (Fig 4). The majority of this reduction was not due to cytotoxicity, indicating a more complex dose response pattern than simple induction. A down-regulation of PRDX6 was not observed in EGCG-fed animals, consistent with serum levels not reaching inhibitory/non-inducing concentrations (13).

Treatment with hydrogen peroxide blocked induction of PRDX6 by EGCG and high doses further reduced PRDX6 levels. These observations are consistent with oxidative stress (at least by this agent) down-regulating PRDX6. Similarly, NAC, a strong antioxidant, failed to induce PRDX6 at 10 μ M, either with or without H₂O₂. These results suggest that EGCG-induced PRDX6 production in salivary gland cells is not depend on its direct antioxidant property, but rather through another mechanism, perhaps mediated *via* the MAPK pathways.

In conclusion, autoimmune diseases such as Sjogren's syndrome and type-1 diabetes are associated with decreased antioxidant defense enzyme levels in affected tissues. The complement of antioxidant enzymes may differ between tissues, but PRDX6 is a common antioxidant enzyme affected by autoimmune reactions in these exocrine glands. Elevation of H₂O₂, and perhaps other ROS species, could potentially be involved in mediating this decrease. EGCG, at a physiological concentration, is able to restore the levels of PRDX6 and other antioxidant defense enzymes, at least in part *via* the MAPK signal transduction pathways, providing protection to the organs from oxidative damage. These novel findings

provide a rationale for future investigations of the use of EGCG or green tea polyphenols to combat autoimmune diseases and development of strategies to delay or prevent the onset of autoimmune diseases.

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Figure 1.

Representative immunostaining of PRDX6 (Panels A, C) and catalase (Panels B, D) expression in submandibular gland epithelium of 22 week old water-fed (Panels A, B), or EGCG-treated (Panels C, D) NOD mice (magnification 200X). A quantitative analysis of submandibular gland samples of 22 week old water and EGCG-treated NOD mice immunostained for PRDX6 (Panel E) and Catalase (Panel F) is shown. Statistical analysis of immuno-positive cells was based on the number of positively stained cells (brown colored) among 1000 cells counted. * Significant difference in comparison to water-treated groups (p<0.05).

Figure 2.

Representative immunostaining of PRDX6 (Panels A, C) and SOD (Panels B, D) expression in the pancreas of 22 week old water-fed (Panels A, B), or EGCG-treated (Panels C, D) NOD mice (magnification 200X). Nuclear staining for both proteins can be clearly observed in EGCG-fed, but not in water-fed NOD mice. A quantitative analysis of submandibular gland samples of 22 week old water and EGCG-treated NOD mice immunostained for PRDX6 (Panel E) and SOD (Panel F) is shown. Statistical analysis of immuno-positive cells was based on the number of positively-stained nuclei (brown colored) among 1000 cells counted. * Significant difference in comparison to water-treated group ($p<0.0001$).

Figure 3.

Panel A: Western analysis of PRDX6 protein expression in HSG cells treated with EGCG at 12, 24 or 48 hours with the indicated concentrations. Panel B: Bar graph shows the quantified mean and standard deviation of results from three independent experiments for each treatment combination (shown by lane number corresponding to Panel A). * Significant difference in comparison to untreated cells ($p < 0.05$).

Figure 4.

Panel A: Western analysis of PRDX6 protein expression in HSG cells treated with EGCG at indicated concentrations, with (denoted by +) or without (denoted by -) inhibitors of JNK or p38. Panel B: Bar graph shows the quantified mean and standard deviation of three independent experiments for each treatment combination (shown by lane number corresponding to Panel A). * Significant change in comparison to untreated cells ($p < 0.05$).

Figure 5.

Panel A: Western analysis of PRDX6 protein expression in HSG cells either treated with 10 μM EGCG or NAC or untreated (denoted by –), with or without hydrogen peroxide at 50 or 100 μM. Bar graph shows the quantified mean of three independent experiments, with standard deviation for each treatment combination (shown by lane number corresponding to Panel A). * Significant difference in comparison to other cell treatments (p<0.05).